Fluorescence Microscopy
Instruments, Methods, Applications

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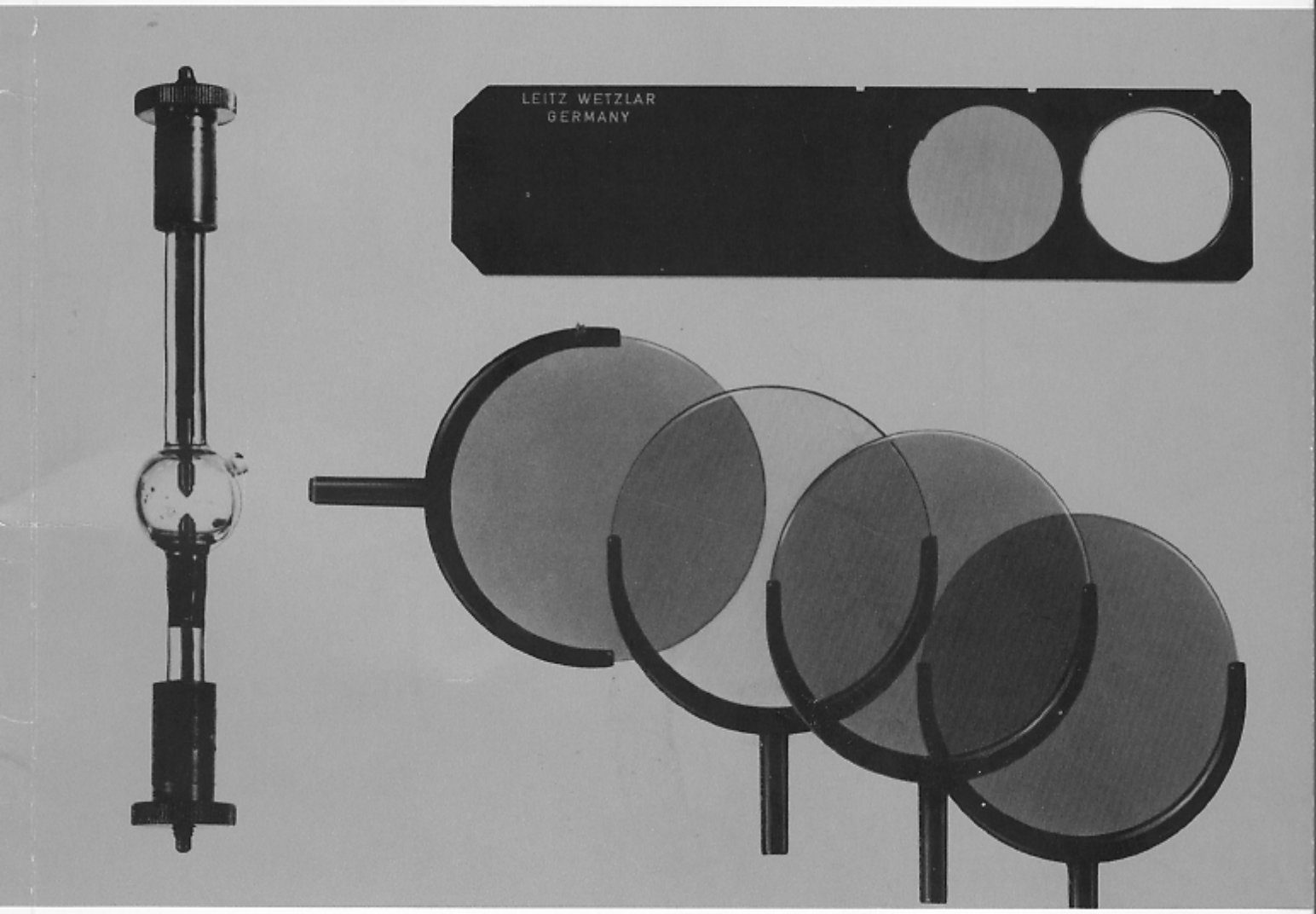

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Preface

This booklet attempts to present the use of fluorescence microscopes in a concise and lucid form according to the latest developments of the instruments in this field, without however, going into a detailed explanation of theoretical and practical principles of fluorescence microscopy.

On the other hand, a comprehensive description is offered of the instruments available and their applications to the various methods of investigation based on fluorescence microscopy from visual observation to fluorescence photomicrography.

The booklet has therefore been written mainly for the user of LEITZ fluorescence microscopes who is already versed in the relevant techniques of preparation and would like to obtain specialized information about the use of fluorescence microscopic devices in his field of research.

A list of references supplements the technical description.

I wish to thank Mrs. Ursula Daun and Mr. Helmut Rühl for their assistance with my investigations of the excitation and fluorescence maxima of the fluorochromes and with the plotting of the filter curves; my thanks are also due to Dr. F. Walter, Head of the Laboratory for Applied Microscopy, for his unstinting help with the production of this brochure.

Karl-Friedrich Koch,
Applied Microscopy Laboratory,
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Wetzlar, February 1972

Contents

I What is Fluorescence?

Certain substances have the property, when irradiated with ultra-violet, violet, blue, or green light, of emitting radiation of their own whose wave length is longer than that of the exciting light (Stoke's Law). This emission phenomenon is called **luminescence:**

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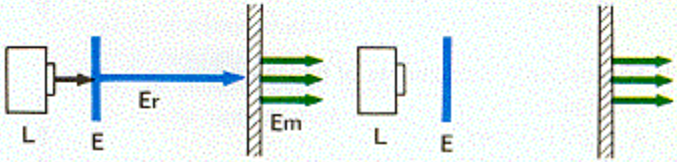
We must however, distinguish between two forms of luminescence:
If after the exciting light is switched off the secondary emission persists for some time at appreciable intensity, we speak of **phosphorescence:**



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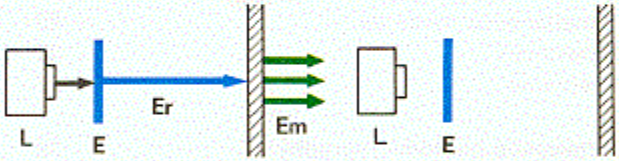
Phosphorescence during irradiation

Phosphorescence after the light source has been switched off

If, however, the excited radiation persists only so long as the exciting light acts on the specimen, we speak of **fluorescence:**



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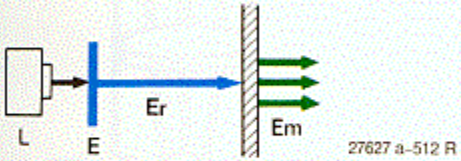
Fluorescence during irradiation only

No fluorescence after the light source has been switched off

Key to symbols:
E = Exciting filter
Er = Exciting light
Em = Emitted light
L = Lamp housing with light source

A Primary fluorescence

A number of unstained specimens have the property of emitting fluorescent light when they are excited with short-wave light. This fluorescence is called **primary** or **auto-fluorescence:**



B Secondary fluorescence through fluorochroming

Many specimens, however, do not exhibit any primary fluorescence. If they are stained with certain dyes — the fluorochromes — tissues that do not appear, but are in fact, different, can be shown in contrasting colours and distinguished by fluorescence.

The light emission caused by fluorochromes is called **secondary fluorescence.**

The dyes acridine orange, auramine, coriphosphin, rhodamine, phosphin 3 R are some of the best known fluorochromes.

Compounds such as the antibiotic tetracycline, which is stored by certain tissues of the organism (bone, teeth) and has fluorescent properties, are fluorochromes in the wider sense.

C Immunofluorescence

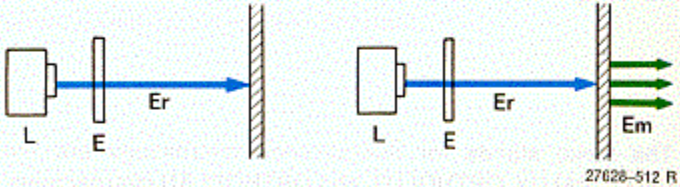
The method of immunofluorescence is highly specific because of its genuine serological reactions. Compounds such as fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) are used as fluorescent labelling substances. These are coupled with the antibodies by co-valent bonds. They are made to react with the corresponding antigen (e.g. pathogenic agent). The antigen-antibody complex produced can be recognized by its fluorescence when it is irradiated with short-wave light.

D Histochemical and cytochemical fluorescent staining

Another method of preparation involves histochemical or cytochemical fluorescent staining. Fluorescent end products are obtained by treatment of the material to be examined with chemical reagents.

The best known method is the process of condensation of biogenic amines (catecholamine, hydrotryptamine) with formaldehyde, developed by ERÄNKÖ (1955) and FALCK (1962).

The technique developed by CASPERSSON et al. (1969, 1970) of cytochemical staining of chromosomes with a mustard gas compound for the demonstration and measurement of DNA distribution also belongs to this category.



Untreated specimen exhibits no fluorescence

Fluorescence after treatment of the specimen (fluorochroming, serological reaction, etc.)

II Microscopic instruments for fluorescence microscopy

Modern fluorescence microscopy relies on the use not only of specific methods of preparation and staining but also of a special fluorescence microscope. In addition the interest of research has recently been focused more and more on fluorometry as a quantitative method through measurement of the intensity of fluorescent radiation.

From these aspects the following stands have been developed for fluorescence microscopy:

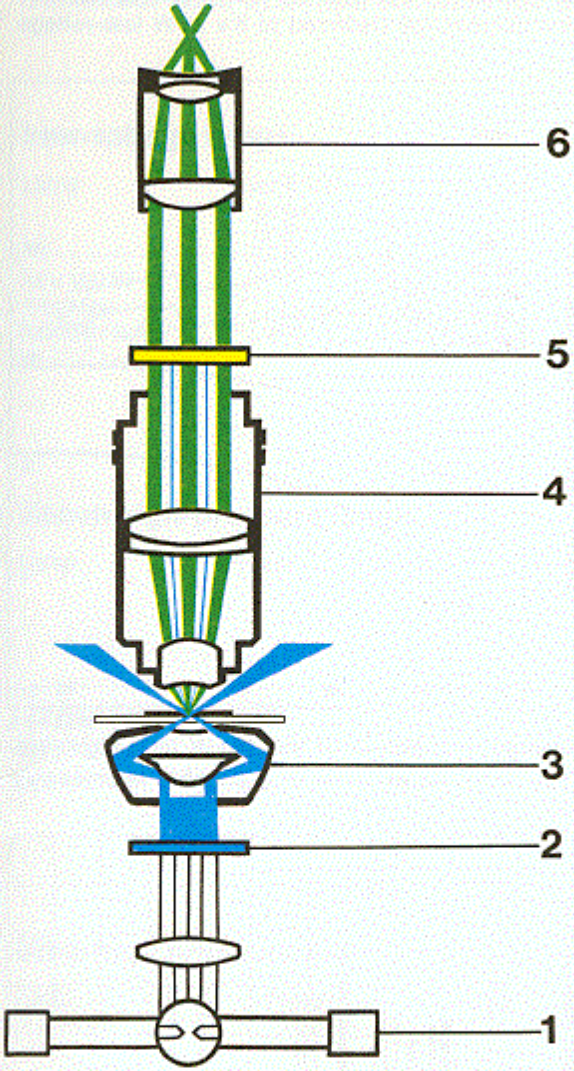
The microscope for routine investigations in diagnostics, preferably for transmitted-light fluorescence microscopy.

The specialized fluorescence microscope for modern incident-light fluorescence excitation and investigation in transmitted light.

The microscope fluorometer, consisting of a research microscope and the MPV microscope photometer as basic instruments, as well as recording and evaluation devices.

The basic stands for fluorescence microscopy are the LEITZ DIALUX, ORTHOLUX, and ORTHOPLAN microscopes.

Microscope outfits for fluorometry are described in Section II/C of this booklet.



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Basic construction of the fluorescence microscope

1 Light source
2 Exciting filter
3 Condenser
4 Objective
5 Suppression filter
6 Eyepiece

The exciting light is produced by a suitable light source. Appropriate exciting filters (primary filters) are inserted in the lamp housing; they transmit only the exciting radiation, which, through a condenser (transmitted-light excitation) or through the fluorescence vertical illuminator (incident-light excitation) reaches the specimen, where it excites fluorescence.

For the observation of the fluorescent radiation emitted by the specimen a suppression filter (secondary filter) matched for the wave length of the fluorescent light must be inserted in the beam. It absorbs (glass filter) or reflects (interference filter) the exciting radiation liable to damage the eye and produces a dark image background.

To concentrate the highest possible amount of exciting energy on the specimen, light source and condenser must be accurately centred. The instructions "Lamp Housing 250 (514-72)" "Lamp Housing 100/100 Z (514-119)" and "Darkground Condensers D 0.80/D 1.20 (513-31)" contain further information.

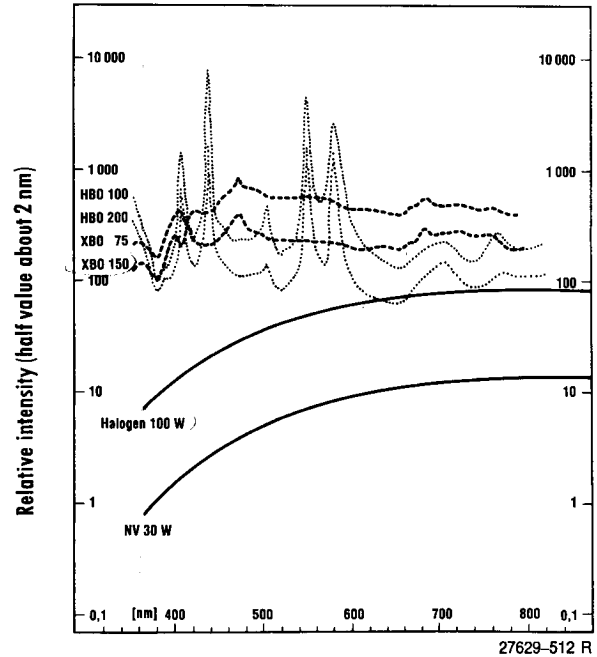
For optimum yield of fluorescence the combination of exciting and suppression filters matched for the specimen under investigation is also important.

A Light sources

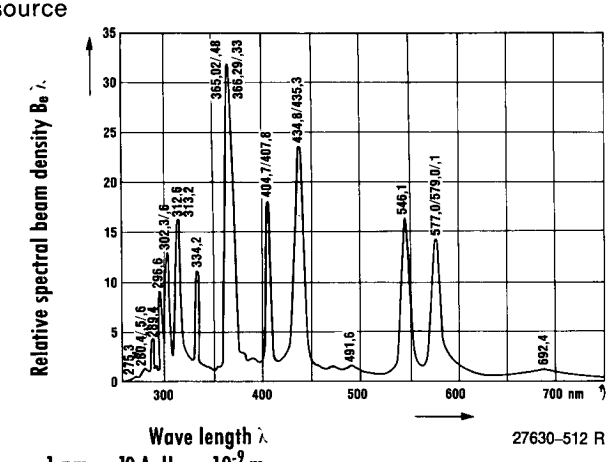
Lamp	Use in incident-light fluorescence	Use in transmitted-light fluorescence	Fluorometry
12 v 100 W tungsten halogen lamp	x (routine, FITC)	x (routine, FITC)	x
75 W high pressure xenon lamp	x	—*)	x
150 W high pressure xenon lamp	x	x	
450 W high pressure xenon lamp	x	x	—
50 W ultra-high pressure mercury lamp	x	x (routine)	—
100 W ultra-high pressure mercury lamp	x	—*)	x
200 W ultra-high pressure mercury lamp	x	x	—

* these lamps are not suitable for transmitted-light excitation, since they do not fill the entrance pupil of the darkground condensers.

Intensity comparison of various light sources suitable for fluorescence microscopy (referred to 6 v 30 W low-voltage lamp)



Spectral energy distribution of the 200 W ultra-high pressure mercury lamp as example of a suitable fluorescence light source



Technical data of lamps suitable for fluorescence microscopy

Low-voltage lamps

Lamp	Electrical power (W)	Voltage (V)	Lamp current (A)	Light flux (lm)	Colour temperature (°K)	Life in hours
12 v 100 W tungsten halogen lamp	100	12	8	2800	3100 at 7.4 amps	ca 50

Xenon high-pressure lamps

Lamp	Power (W)	Voltage (V)	Lamp current (A)	Light flux (lm)	Mean luminous density (sb)	Dimensions of the illuminated field (mmLxB)	Colour temperature (°K)	Life in hours
75 W	75	14	5,4	1 000	40 000	0,5 x 0,25	6000	ca. 400
150 W	150	20	7,5	3 000	15 000	2,2 x 0,5	6000	ca. 1200
450 W	450	18	25	13 000	35 000	2,7 x 0,9	6000	ca. 2000

Ultra-high pressure mercury lamps

Lamp	Power (W)	Voltage (V)	Lamp current (A)	Light flux (lm)	Mean luminous density (sb)	Dimensions of the illuminated field (mmLxB)	Life in hours
50 W	50	L1 39..45 L2 34..39	1,3 1,45	2000	30 000	1,0 x 0,3	ca. 100
100 W	100	20	5	2200	170 000	0,25 x 0,25	ca. 200
200 W	200	L1 61 ± 4 L2 53 ± 4	3,4–3,9 3,9–4,4	9500	33 000	2,2 x 0,5	ca. 200

Light sources in colour photography

Fluorescence colour photography:	All light sources are suitable (daylight colour film)
Other microscopic methods:	for artificial-light colour film 12 v 100 W tungsten halogen lamp
	For daylight colour film high-pressure xenon lamps 75 W 150 W 450 W

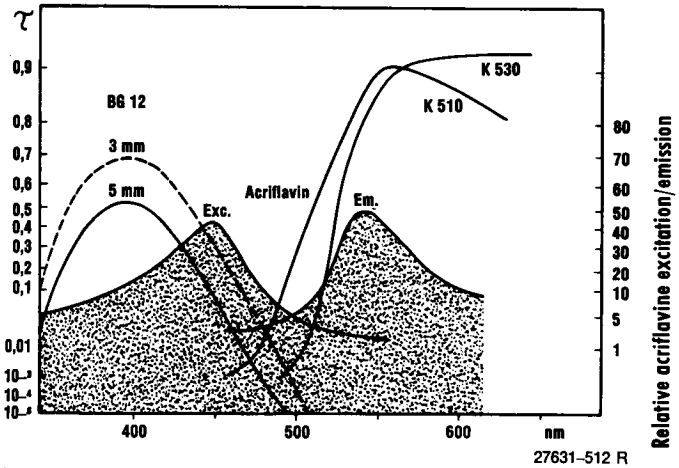
Except in fluorescence colour photography the ultra-high pressure mercury lamps are not suitable for use with colour film.

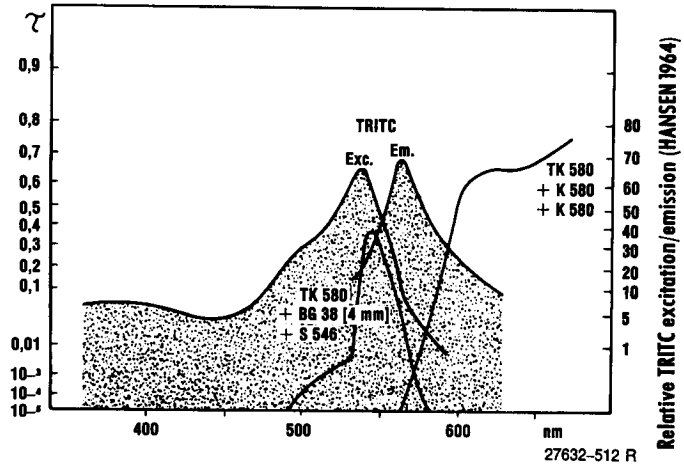
B Filters

Not only the choice of a suitable light source, but also that of the filters and their combination depends on the nature of the specimen. Both must be matched for the fluorescence staining method used. This requires knowledge of the spectral values for the excitation and fluorescence (emission).
We generally distinguish between filter combinations for broad-band, narrow-band, and selective excitation.

Broad-band excitation

Broad-band fluorescence constitutes the classical method. Here the exciting light is filtered out with the aid of dyed glass filters (UG filters, BG filters) of great half-value width and the fluorescent light observed from a certain wave length onwards through suppression filters.
The method is used in transmitted light as well as in incident light wherever the excitation and emission maxima of the fluorochrome are widely separated. With the aid of the broad transmission ranges of the exciting filters the fluorescence can be excited with sufficient energy in or near the maximum without the emitted fluorescent light being influenced by the exciting radiation. This means that the observer can use a suppression filter which, because of the position of the edge, practically completely suppresses the exciting radiation, but fully transmits the maximum of the fluorescent radiation and therefore the "true" colour of the fluorescent light.
The illustration shows the example of a typical case of acriflavine fluorescence.





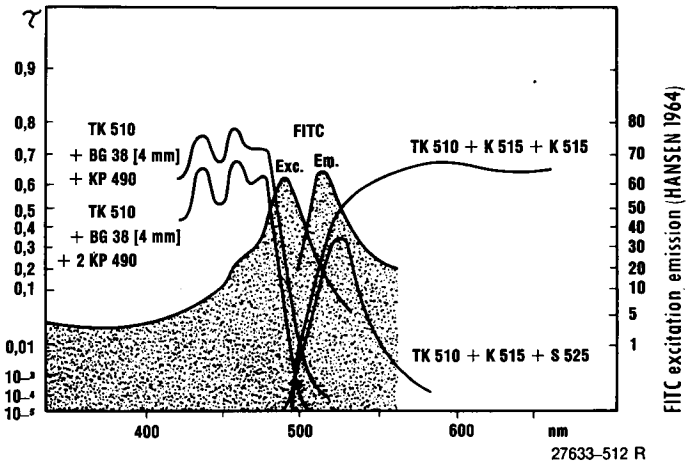
The maxima of excitation and emission are separated by about 100nm. Thus a BG 12 (32mm) exciting filter and K 510 or K 530 suppression filter can be chosen as combination for effective broad-band fluorescence.

Narrow-band excitation

Narrow-band excitation is used wherever the excitation and emission maxima of fluorescence are closely spaced. Here the exciting light is filtered out with interference band filters of small half-value width if necessary combined with conventional glass filters. This permits the excitation of fluorescence in a narrow range around the excitation maximum, without disturbance of the fluorescence emission by exciting light.

The usual edge filters are used as suppression filters; for the strict selection of fluorescence within the range of the emission maximum interference selection filters are used. The illustration above shows an example of narrow-band fluorescence. It illustrates the situation during excitation of red fluorescence of tetra-methyl rhodamine isothiocyanate (TRITC) by green light of 546nm.

The TRITC maxima are so closely spaced here that the primary filter combination of the S 546 interference band filter (type AL) with the BG 38 and BG 36 glass filters becomes necessary to cut off the exciting radiation within the range of the emission maximum of TRITC. The graph further demonstrates that, because of the position of the maxima and the properties of the primary filter combination it is not the yellow emission maximum, but an intense red-orange or red fluorescence (PLOEM 1969) that can be



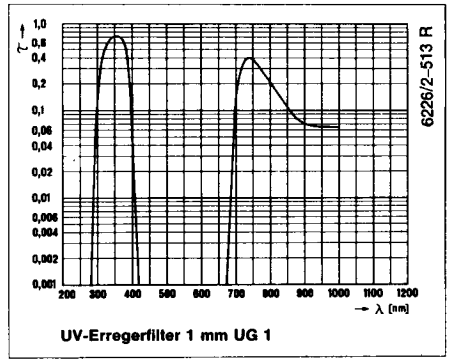
observed, since a K 590 or K 610 suppression filter must be inserted.

Selective excitation

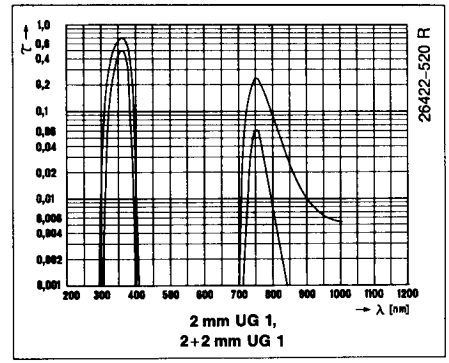
Similar to narrow-band excitation, selective excitation is used wherever excitation and emission maxima of the fluorescence are closely spaced. It differs from narrow-band excitation in that asymmetrical interference filters with a particularly steep cut-off are used. These filters have their maximum transmission in the excitation maximum of the fluorescent dye. Immediately next to the maximum their transmission is strictly limited in the direction of the longer wave length by the cut-off. It is combined on the observer's side with the usual edge suppression filters or with interference selection filters. Selective excitation of FITC immunofluorescence is the most widely used at the moment. With the KP 490 interference filter the excitation is produced directly within the range of the excitation maximum of FITC (about 490mm). For the observation of the characteristic apple green FITC fluorescence the K 510 edge filter, and particularly the S 525 selection filter is used for suppression. The additional BG 38 colour glass filter prevents the low transmission of the KP filter in the red from becoming visible.

Filter curves

Filters for UV excitation

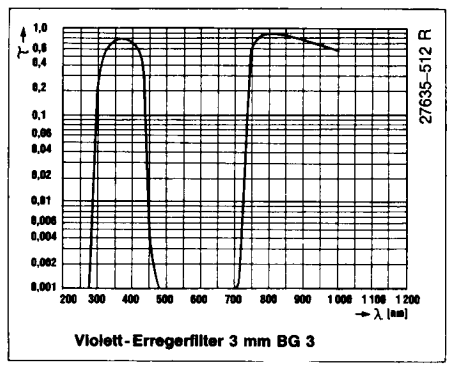


1mm UGI UV exciting filter

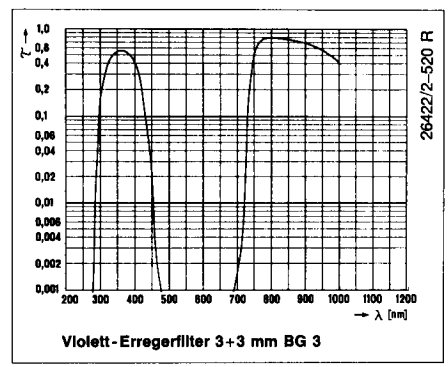


2mm UG 1,
2+2mm UG 1

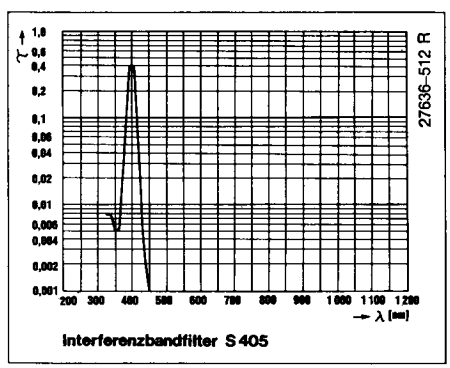
Filters for violet excitation



3mm BG 3 violet exciting filter

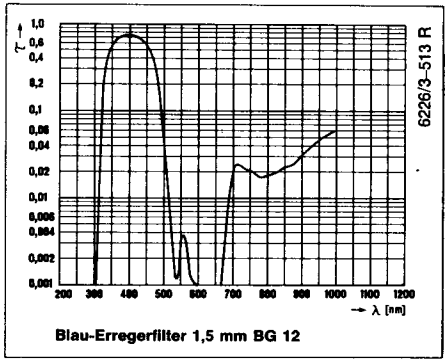


3+3mm BG 3 violet exciting filter

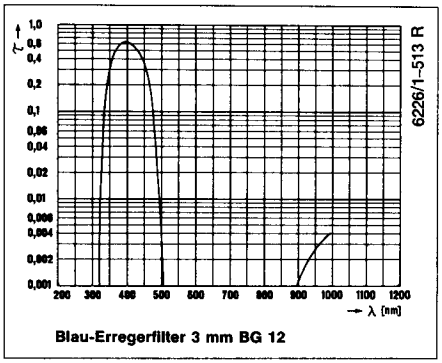


S 405 Interference band filter

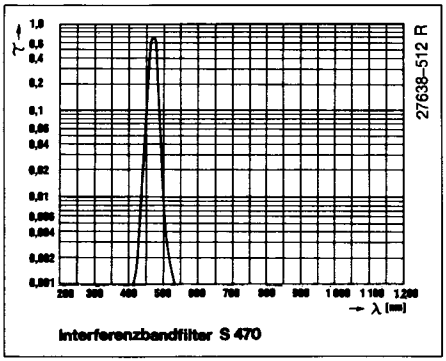
Filter for blue excitation



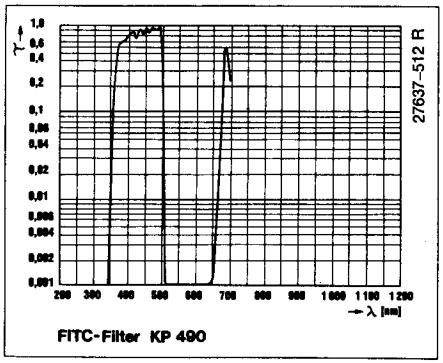
1.5mm BG 12 blue exciting filter



3mm BG 12 blue exciting filter

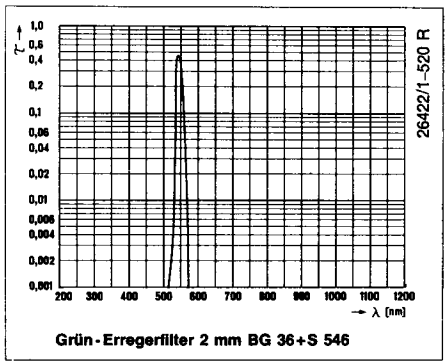


S 470 interference band filter



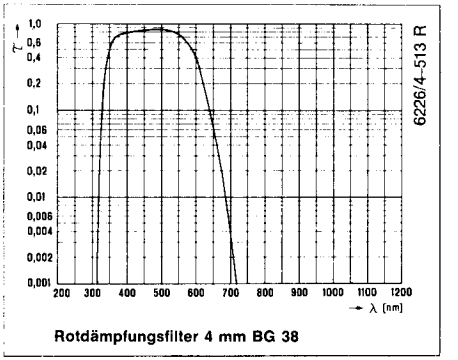
KP 490 FITC filter

Filter for green excitation



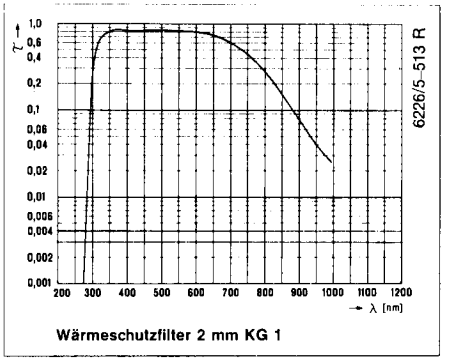
2mm BG 36+S 546 green exciting filter

Red suppression filter



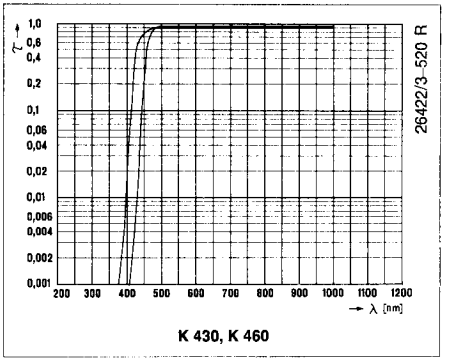
4mm BG 38 red suppression filter

Heat filter

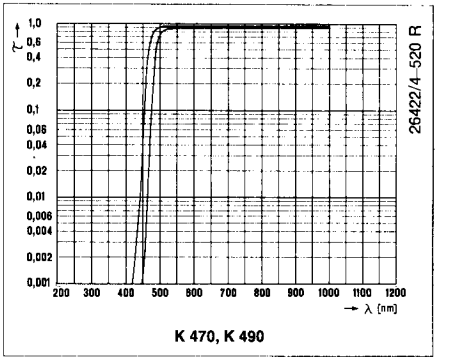


2mm KG 1 heat absorption filter

Suppression filter

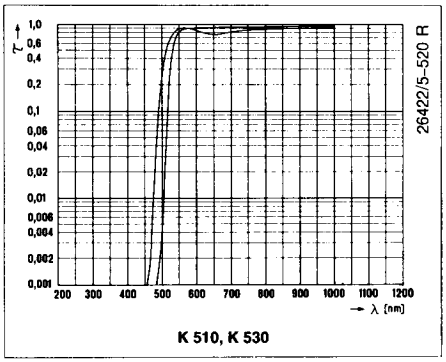


K 430, K 460

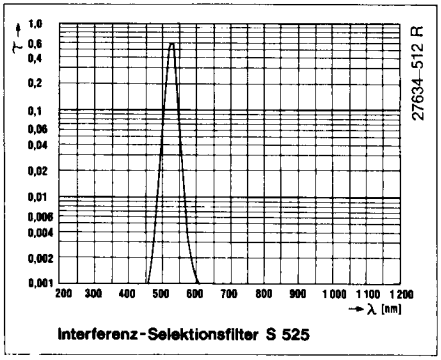


K 470, K 490

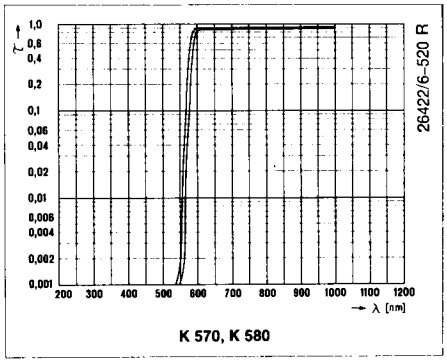
Suppression filter



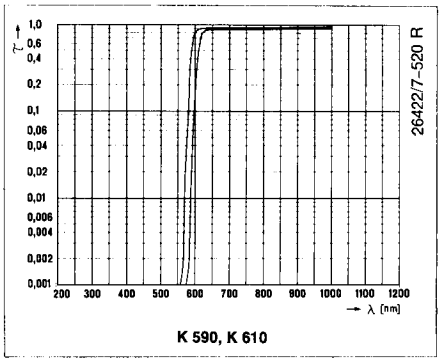
K 510, K 530



S 525 Interference selection filter



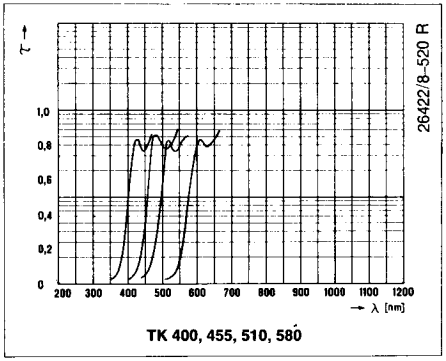
K 570, K 580



K 590, K 610

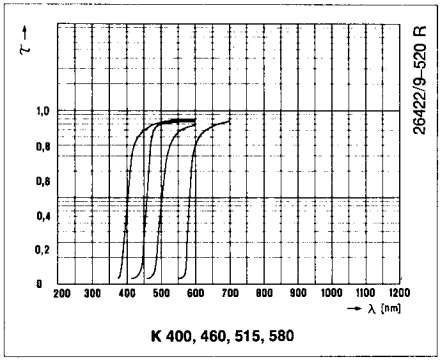
Filter for fluorescence vertical illuminator

Dichroic beam splitting mirrors



TK 400, 455, 510, 580

Built-in suppression filters



K 400, 460, 515, 580

Use of the fluorescent dyes

Fluorochrome	Objects to be stained	Concentration and Solution		Staining time
Diamino naphtylsulphonic acid	Technique see R. C. NAIRN: Fluorescent Protein Tracing. E. & S. Livingstone Ltd., Edinburgh & London 3rd Edition 1969. Also contains further references.			
Berberin sulphate	Cell nuclei	1:1,000	Aqueous	10 sec
	Bacteria	1:1,000	Aqueous	15–35 sec
Catecholamine	For technique see FALCK B., N. A. HILLARP, G. THIEME and A. TORP: Fluorescence of catecholamines and related compounds condensed with formaldehyde. Journ. Histochem. and Cytochem. 10 (1962), 348.			
Euchrysin	Cell Nuclei	1:10,000	Aqueous	3 min
	Leucocytes	1:1,000	Aqueous	4 min
	Lymphocytes			
	Mucus	1:20,000	Aqueous	5 min
	Wood	1:1,000	Aqueous	15 sec
Primulin O	Wood	1:500	Aqueous	1 min
	Elementary virus bodies	1:1,000	Aqueous	20 sec
Pyronin	Techniques see H. C. BURCK: Histolog. Technik (1966), 123–124 Georg Thieme Verlag, Stuttgart.			
Quinacrine mustard	For techniques see CASPERSSON T., L. ZECH, E. J. MODEST, G. E. FOLEY, U. WAGH and E. SIMONSSON: Chemical Differentation with Fluorescent Alkylating Agents in VICIA FABA Metaphase Chromosomes. Experimental Cell Research 58 (1969), 128–140.			
Thioflavine S	Leucocytes Lymphocytes	1:1,000	Aqueous	4 min
Aurophosphin G	Mucus	1:20,000	Aqueous	5 min
Acridine yellow	Tubercle bacilli	1:500	Aqueous	30 sec
Acridine orange	Cell nuclei	1:10,000	Aqueous	3 min
	Mucus	1:20,000	Aqueous	5 min
	Vacuoles	1:5,000	Alkaline	5–10 min
	Bacteria	1:30,000	Physiological saline solution	

Use of the fluorescent dyes

Fluorochrome	Objects to be stained	Concentration and Solution		Staining time
Auramine	Tubercle bacilli	1:1,000	Aqueous	10 min
Coriphosphin O	Cell nuclei	1:10,000	Aqueous	3 min
	Leucocytes	1:1,000	Aqueous	4 min
	Lymphocytes			
	Mucus	1:20,000	Aqueous	5 min
	Cell walls	1:1,000	Aqueous	1 min
	Wood	1:1,000	Aqueous	1 min
	Diphtheria bacteria	1:1,000	Aqueous	10 min
Fluorescein isothiocyanate (FITC)	For technique see R. C. NAIRN: Fluorescent Protein Tracing.			
Phosphin 3 R	Cell nuclei	1:10,000	Aqueous	3 min
Tetracycline	For technique see EGER W., F. GÖTZ and H. KÄMMERER: Herstellung von Dünnschliffen aus Knochen und Weichgeweben nach Markierung mit Tetracyclinen". Langenbecks Arch. Klin. Chir. 306 (1964), 205–214.			
Lissamine-Rhodamine B (RB 200)	For technique see R. C. NAIRN: Fluorescent Protein Tracing.			
Magdala red	Cell walls	1:1,000	Aqueous	1 min
Pararosaniline (Feulgen)	For technique see B. ROMEIS: Mikroskopische Technik 16th Edition (1968) 293–297.			
Rhodamine B	Vacuoles	1:1,000	Aqueous	
Acid Fuchsin	For technique see H. M. FROST: Bone remodelling dynamics. Ch. C. Thomas Springfield, USA-Illinois (1963).			
Thiazin red R	Albumin (together with euchrysin)	1:10,000	Aqueous	½ min
Tetra-methyl rhodamine isothiocyanate (TRITC)	For technique see R. C. NAIRN: Fluorescent Protein Tracing.			

Fluorescence-free embedding media

Embedding medium	Refractive index n_D
Entellan	1.501
Eukitt	1.491
Non-fluorescent immersion oil	1.515
Liquid paraffin	1.480

To demonstrate primary fluorescence the specimen is first excited with ultra-violet light and observed through various suppression filters. If this does not produce results, continue examination with other exciting radiations according to the table.
The filter combination to be used in connection with the microscope outfits listed on pp. 28 often depends on the problem to be solved or the aim of the investigation. For this reason no fixed filter combinations for the individual fluorescent dyes are given here, but only the required exciting radiation.
For excitation one of the filter combinations associated with the required exciting radiation listed on p. 20 is used.

* Manufacturers' value. The other values were measured in stained specimens with the LEITZ microspectrograph.

Excitation and emission of the fluorescent dyes

Fluorochrome	Exciting radiation	Mean exciting wave length (nm)	Mean wave length of fluorescence (nm)
Diamino naphthylsulphonic acid (DANS)*	Ultra-violet	340	525
5-hydroxy tryptamine (5-HT)*		380–415	520–530
Aurophosphin G	Violet/ blue	450	580
Berberin sulphate		430	550
Catecholamine		410	470
Euchrysin		430	540
Primulin O		410	550
Pyronin		410	540
Quinacrine mustard*		430–460	490–530
Thioflavine S		430	550
Acridine yellow	blue	470	550
Acridine orange		470	530–650
Auramine		460	550
Coriphosphin O		460	575
Fluorescein isothiocyanate (FITC)*		490	520
Morin			
Phosphin 3 R		465	565
Tetracycline		390	440
Lissamine-Rhodamine B (RB 200)*	Green	575	595
Magdala red		540	570
Pararosaniline (Feulgen)		570	625
Rhodamine B		540	625
Acid fuchsin		540	630
Thiazin red R		510	580
Tetramethyl rhodamine isothiocyanate (TRITC)*		540	570

Filter combinations (as per February 1972) +

Exciting radiation	Broad-band transmitted light	Broad-band incident light	Narrow-band incident light	Selective excitation	Setting wheel on the illuminator	Dichroic beam splitting mirrors/built-in suppression filters	Suppression filters
Ultra-violet	1mm UG 1 2mm UG 1	2mm UG 1	2x2mm UG 1		1	TK 400 / K 400	from K 430 onwards corresponding to the fluorescence maximum
Violet	3mm BG 3	(2x) 3mm BG 3	3mm BG 3 +S 405 (Type AL)	KP 425 +3mm BG 3	2	TK 455 / K 460	from K 460 onwards according to the fluorescence maximum
Blue	1.5mm BG 12 3mm BG 12 2x3mm BG 12	3mm BG 12	1.5mm BG 12 +S 470 (Type AL)	**FITC** transmitted light K 490 **FITC** incident light 2xKP 490** (+K 480)***	3	TK 510 / K 515	from K 510 onwards according to the fluorescence maximum for FITC*: K 510 or S 525 Type AL
Green			2mm BG 36 +S 546 (Type AL)	KP 560 +2mm BG 36	4	TK 580 / K 580	from K 580 onwards corresponding to the fluorescence maximum K 610 for TRITC*

With all filter combinations a 2mm KG 1 heat filter and a 4mm BG 38 red suppression filter must be inserted in the lamp housing. Filters inserted in the lamp housing are exposed to strong heat. It is therefore recommended to swing them out when they are not in use.

* With the two-wave-lengths excitation of FITC/TRITC double staining a suppression filter slide with the K 510 and K 610 or S 525 and K 610 filters side by side must be used.

** 2 KP 490 = KP 500

*** K 480 can be inserted in the lamp housing to eliminate autofluorescence excited by the UV portion of KP 490.

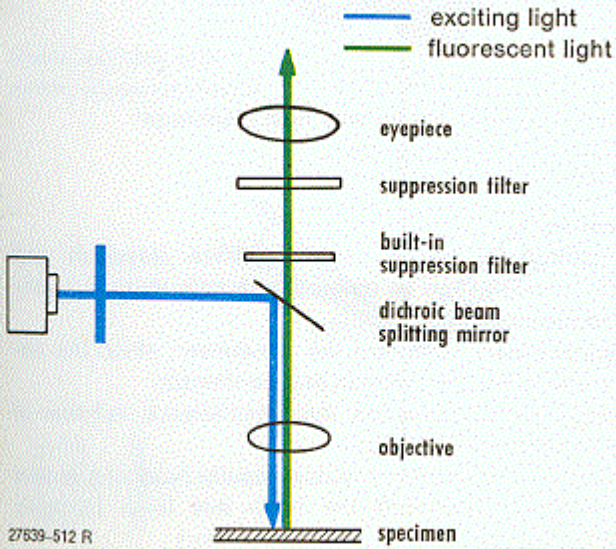
\+ If the filter combinations or designations are changed an insert will be published for the filter table.

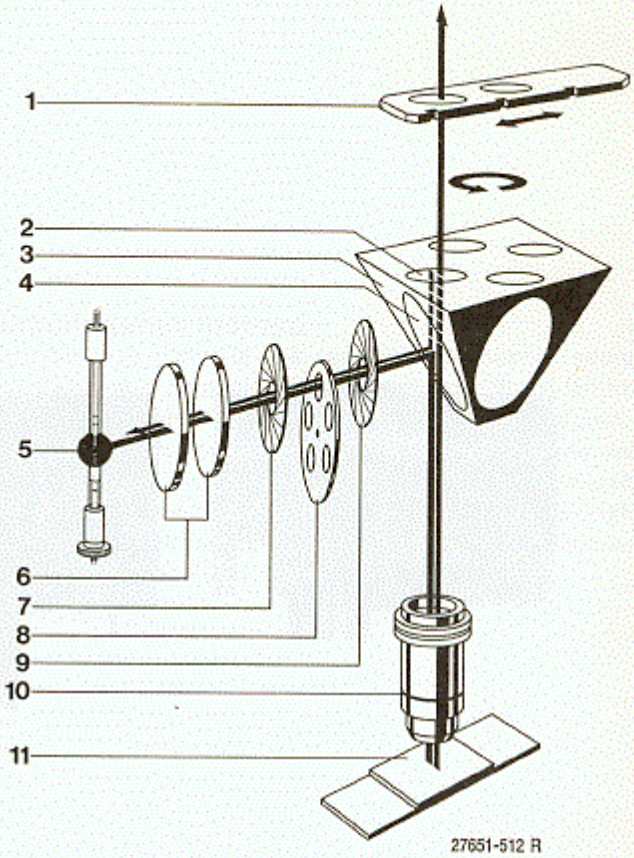
C Illuminating methods in fluorescence microscopy

Incident-light fluorescence

With fluorescence excitation with incident light the exciting radiation is directed at the specimen from above through an incident-light illuminator with dichroic beam splitting mirrors through the microscope objective. Here the full aperture of the objective is utilized for excitation. This method guarantees a particularly high fluorescence intensity because no light losses can occur through scattering or primary absorption in the specimen, which is possible with transmitted-light excitation.

Since the intensity of the exciting radiation depends on the numerical aperture of the objective used, microscope objectives of the largest possible aperture should be used for incident-light excitation.

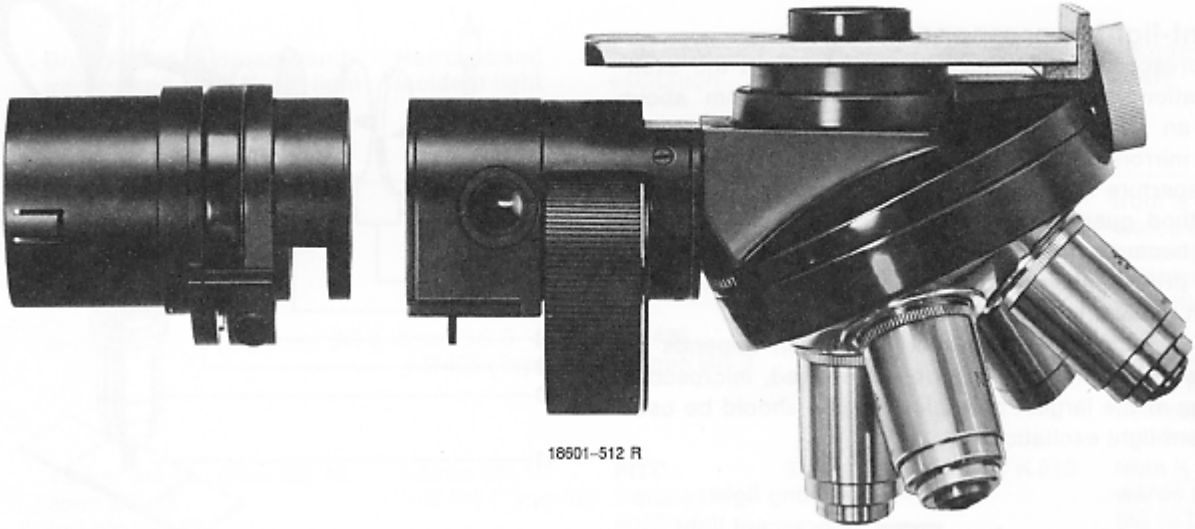




Fluorescence vertical illuminator according to Ploem

1 Suppression filter slide
2 Suppression filter in the fluorescence illuminator
3 Revolving turret with dichroic beam splitting mirrors and suppression filters
4 Dichroic beam splitting mirrors
5 Light source
6 Filter in the lamp housing
7 Field diaphragm
8 Exciting filter turret
9 Stray-light stop
10 Objective
11 Specimen

Later fluorescence vertical illuminators are equipped with an exciting filter turret (cf. illustration); this permits instant change of exciting filters.



Fluorescence vertical illuminator with exciting filter turret and filter holder with field diaphragm

For fluorometry incident-light illuminators with lateral mirror are used.
Built-in filters:
Position 1: 2mm UG 1
Position 2: 3mm BG 3 + S 405 interference band filter
Position 3: 2x KP 490 (= KP 500)
Position 4: 2mm BG 36 + S 546 interference band filter
Position 5: Empty aperture; use of any desired filter combinations possible.

Application
Investigations in biology and medicine (research and routine), investigation of opaque materials (natural sciences and technology).
It is unnecessary to centre the condenser, since the objective also fulfils the function of a condenser.
The objective aperture has the simultaneous function of field and aperture diaphragm.
Focusing on the specimen automatically produces quality of illumination (provided the lamp has been correctly centred).

Transmitted-light fluorescence

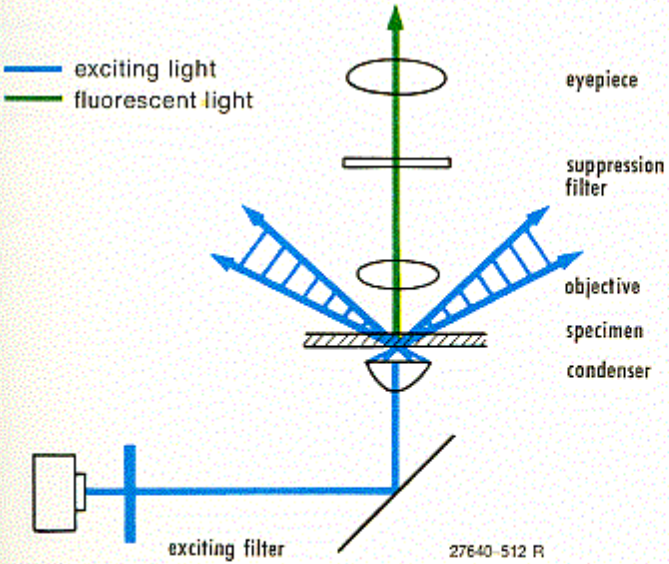
Fluorescence excitation with transmitted light is the classical method. It is carried out with darkground condensers or brightfield condensers.

Darkground condensers have the advantage that no exciting light reaches the objective and thus a dark background for the specimen is guaranteed. However, only objectives of apertures up to 1.15 can be used.

Brightfield condensers permit the use of objectives of larger aperture. But the image contrast is inferior, since here the exciting light also reaches the objective.

Simultaneous method

Simultaneous methods are used in fluorescence microscopy whenever besides the fluorescent structures the non-fluorescent parts of the object, too, are to be shown in brightfield, darkground, phase contrast or polarized light. The use of simultaneous methods is particularly favourable in connection with the fluorescence vertical illuminator, since here bright and contrasty fluorescence is preserved in the brightened field of view of the microscope.



Fluorescence microscopy with darkground condensers

Application

Investigation in biology and medicine (routine and research)

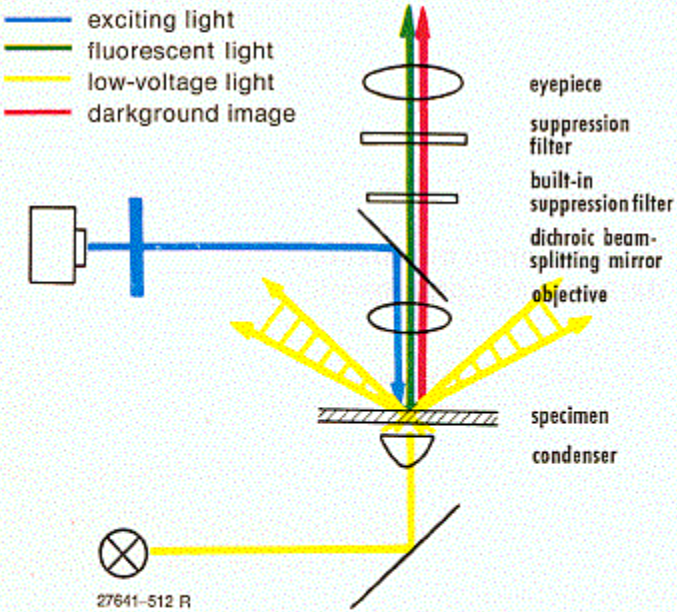
Routine FITC fluorescence with KP 490 exciting filter and K 510 suppression filter.

Simultaneous incident-light and transmitted-light fluorescence.

Advantages over the brightfield condenser:

Dark background. Use of objectives with weak primary fluorescence possible.

Transmitted-light darkground / incident-light fluorescence

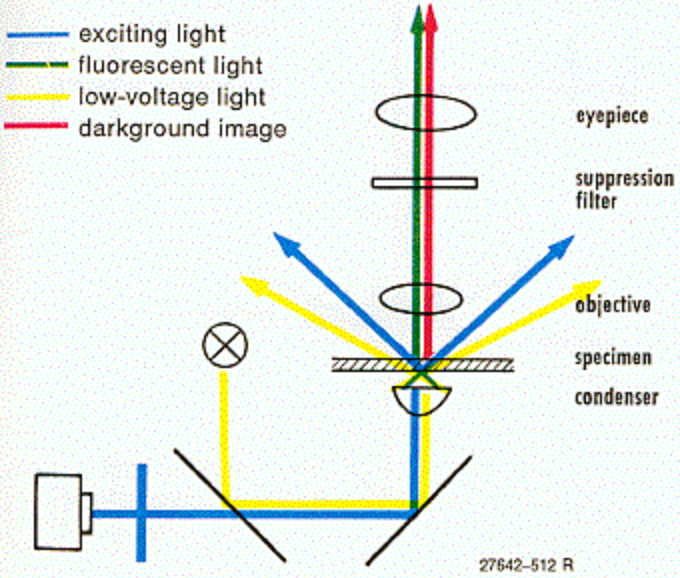


Applications

In biology and medicine

For the demonstration of the structure of the tissue surrounding the fluorescent parts of the object and for the localization of the fluorescent particles in tissue.
For the simultaneous demonstration of fluorescent and non-fluorescent object details whose colours can be also differentiated.
For the assessment of the number of non-fluorescent compared with that of the fluorescent particles.

Transmitted-light darkground / transmitted-light darkground fluorescence



Applications
In biology and medicine

For the demonstration of the structure of tissue surrounding the fluorescent object parts and localization of the fluorescent particles in tissue.
For the simultaneous demonstration of fluorescent and non-fluorescent object details whose colours can also be differentiated.
For the assessment of the number of non-fluorescent compared with that of fluorescent particles.

Incident-light fluorescence / transmitted-light phase contrast

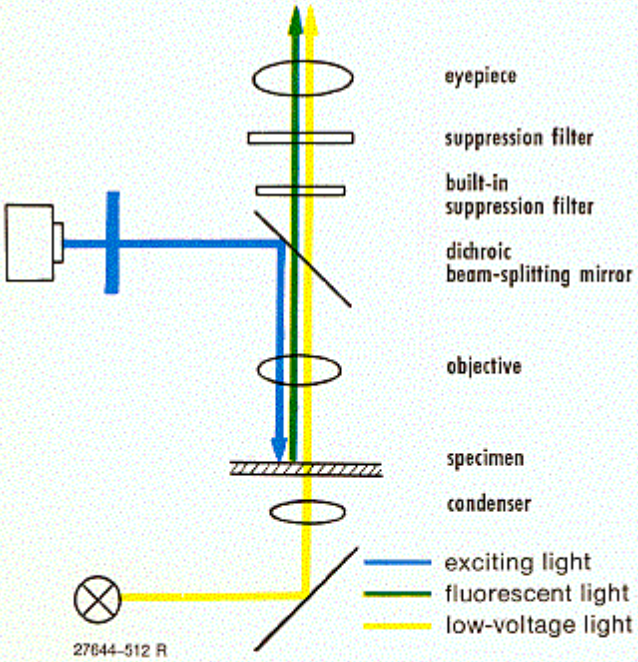
Applications

In biology and medicine

For the demonstration of the structure of the tissue surrounding the fluorescent object parts.

For the localization of the fluorescent particles in tissue.

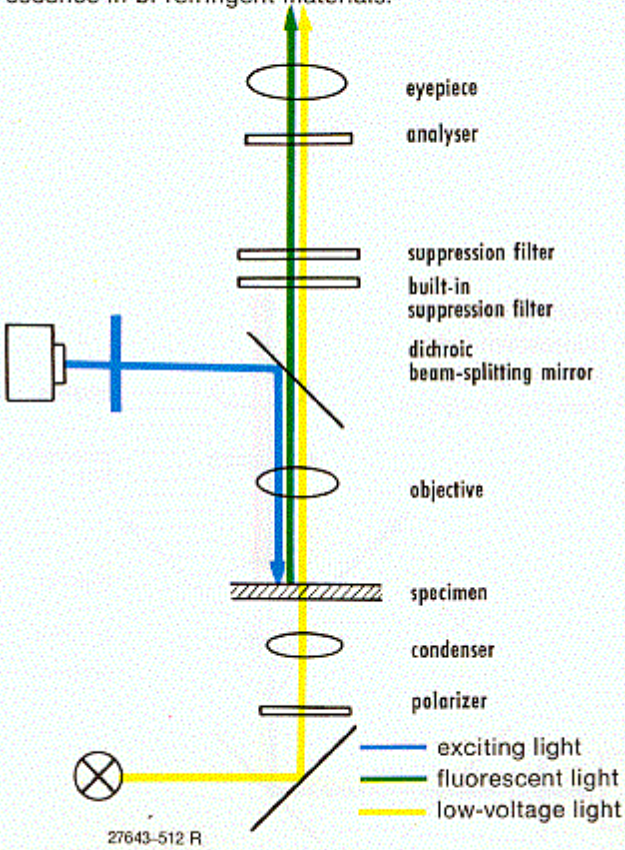
For the assessment of the number of non-fluorescent compared with that of the fluorescent particles.



Incident-light fluorescence / transmitted polarized light

Applications

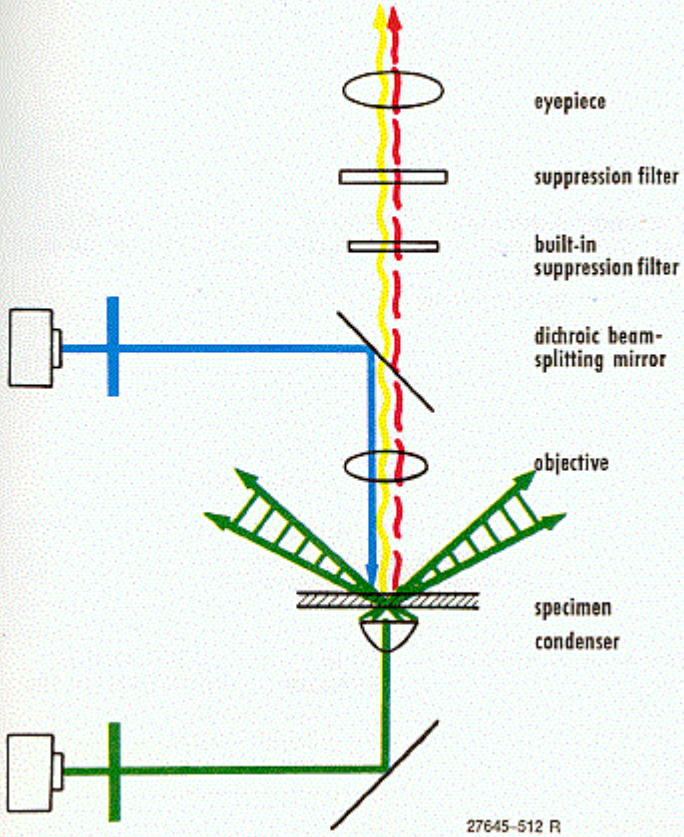
Simultaneous demonstrations of polarization and fluorescence in bi-refringent materials.



Simultaneous incident- and transmitted light fluorescence

Applications

Demonstration of two object portions with different fluorochromes (e.g. transmitted-light blue excitation and incident-light green excitation).



- incident exciting light
- transmitted exciting light
- fluorescent light caused by incident-light excitation
- fluorescent light caused by the transmitted-light excitation

Incident-light fluorescence microscopes

Microscope	Light source	Base Mirror housing	Filter*	Illuminator condenser	Objectives	Tube	Eyepieces
DIALUX Routine	Lamp housing 100 Z with 50 W mercury lamp	Lamp holder	BB SA	Fluorescence vertical illuminator	Achromats, fluorite systems	S FSA	PERIPLAN® 6.3x PERIPLAN GF 10x
ORTHOLUX® ORTHOLUX II ORTHOPLAN Routine	Lamp housing 100 Z with 50 or 100 W mercury lamp;	ORTHOLUX: base A with mirror housing 30 S	BB SA	Fluorescence vertical illuminator according to PLOEM	Achromats, fluorite systems	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x
	Lamp housing 250 with 200 W mercury lamp;	ORTHOLUX II: mirror housing 250/0					
	ORTHOPLAN: lamp housing 500 with 200 W mercury lamp	ORTHOPLAN: mirror housing 250 S mirror housing 500					
DIALUX ORTHOLUX ORTHOLUX II ORTHOPLAN Research	Lamp housing 100 Z with 100 W mercury lamp or 75 W xenon lamp	ORTHOLUX: base A with mirror housing 30 S	SB SA	Fluorescence vertical illuminator	Achromats, fluorite systems, apochromats	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x
	Lamp housing 250 with 200 W mercury lamp or 150 W xenon lamp	ORTHOLUX II: mirror housing 250/0 DIALUX: lamp holder					
	ORTHOPLAN: Lamp housing 500 with 200 W mercury lamp 150 or 450 W xenon lamp	ORTHOPLAN: mirror housing 250 S mirror housing 500					
ORTHOLUX ORTHOLUX II ORTHOPLAN Fluorometry (MPV)	Lamp housing 100 Z with 100 W mercury lamp or 75 W xenon lamp	ORTHOLUX/ ORTHOLUX II: ARISTOPHOT®: ORTHOPLAN®: camera rail	SB SA	Fluorescence vertical illuminator according to PLOEM with lateral mirror	Achromats, fluorite systems, apochromats	FS or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x

* BB = Broad-band excitation SB = Narrow-band excitation SA = Selective excitation

Microscopes for fluorescence microscopy with darkground condenser

(Routine, research, fluorometry)

Microscope	Light source	Base Mirror housing	Filter	Condenser	Objectives	Tube	Eyepieces
DIALUX® routine (FITC)	12 v 100 W tungsten halogen lamp in lamp housing 100 or 100 Z	Feet with adapter	KP 490 + 4mm BG 38 / K 510	Darkground condenser D 0.80 or D 1.20	Achromats, fluorite systems	S FSA	PERIPLAN 6.3x PERIPLAN GF 10x
Routine (Broad band)	Lamp housing 100 Z with 50 W mercury lamp or lamp housing 250 with 200 W mercury lamp	Microscope base L or feet with adapter (only for lamp housing 100 Z)	BB	Darkground condenser D 0.80 or D 1.20	Achromats, fluorite systems	S FSA	PERIPLAN 6.3x PERIPLAN GF 10x
ORTHOLUX ORTHOLUX II ORTHOPLAN Routine (FITC)	Lamp housing 100 Z with 50 W mercury lamp; lamp housing 250 with 200 W mercury lamp or 150 W xenon lamp ORTHOPLAN: lamp housing 500 with 150 or 450 W xenon lamp	ORTHOLUX: base A with mirror housing 30 S ORTHOPLAN: mirror housing 250 S mirror housing 500 ORTHOLUX II: Mirror housing 250/0	KP 490 + 4mm BG 38 / K 510 or S 525	Darkground condenser D 0.80 or D 1.20	Achromats, fluorite systems	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x ORTHOPLAN PERIPLAN 6.3x GW/GG 8x, GW 10x
ORTHOLUX ORTHOLUX II ORTHOPLAN Routine (Broad band)	Lamp housing 100 Z with 50 W mercury lamp Lamp housing 250 with 200 W mercury lamp ORTHOPLAN: Lamp housing 500 with 200 W mercury lamp	ORTHOLUX: Base A with mirror housing 30 S ORTHOPLAN: mirror housing 250 S mirror housing 500 ORTHOLUX II: Mirror housing 250/0	BB	Darkground condenser D 0.80 or D 1.20	Apochromats, achromats fluorite systems	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x ORTHOPLAN: PERIPLAN GW 6.3x GW/GG 8x, GW 10x

Microscope for fluorescence microscopy with darkground condenser

(Routine, research, fluorometry)

Microscope	Light source	Base Mirror housing	Filter	Condenser	Objectives	Tube	Eyepieces
Research	Lamp housing 250 with 200 W mercury lamp or 150 W xenon lamp (FITC) ORTHOPLAN: Lamp housing 500 with 200 W mercury lamp, 150 or 450 W xenon lamp	ORTHOLUX: Base A with mirror housing 30 S ORTHOLUX II: Mirror housing 250/0 ORTHOPLAN: Mirror housing 250 S mirror housing 500	BB SB SA	Darkground condenser D 0.80 or D 1.20	Apochromats, fluorite systems, achromats	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x ORTHOPLAN: PERIPLAN GW 6.3x GW/GG 8x, GW 10x
Fluorometry (MPV)	Lamp housing 250 with 150 W xenon lamp (if necessary for FITC lamp housing 100 Z with 12 v 100 W tungsten halogen lamp)	ORTHOLUX/ ORTHOLUX II: ARISTOPHOT: ORTHOPLAN: camera rail	SB SA	Darkground condenser D 0.80 or D 1.20	Apochromats, fluorite systems	FS or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x ORTHOPLAN: PERIPLAN GW 6.3x GW/GG 8x, GW 10x

Fluorescence Microscopes for transmitted-light darkground/incident-light fluorescence

(Routine and research)

Microscope	Light source	Base Mirror housing	Filter	Illuminator condenser	Objectives	Tube	Eyepieces
DIALUX Routine	Lamp housing 100 Z with 50 W mercury lamp	Lamp holder	BB SA	Fluorescence vertical illuminator	Achromats, fluorite systems	S FSA	PERIPLAN 6.3x PERIPLAN GF 10x
ORTHOLUX ORTHOLUX II ORTHOPLAN Routine	Lamp housing 100 Z with 50 W mercury lamp Lamp housing 250 with 200 W mercury lamp or 150 W xenon lamp (FITC) ORTHOPLAN: Lamp housing 500 with 200 W mercury lamp	ORTHOLUX: base A with mirror housing 30 S ORTHOLUX II: Mirror housing 250/0 ORTHOPLAN Mirror housing 60 S (incident light) mirror housing 500	BB	Fluorescence vertical illuminator according to PLOEM and darkground condenser D 0.80 or D 1.20	Achromats, fluorite systems	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x
DIALUX ORTHOLUX ORTHOLUX II ORTHOPLAN	Lamp housing 100 Z with 100 W mercury lamp or 75 W xenon lamp	ORTHOLUX: Base A with mirror housing 30 S	BB SB SA	Fluorescence vertical illuminator and dark-ground condenser D 0.80 or D 1.20	Achromats, fluorite systems Apochromats	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x
Research	Lamp housing 250 with 200 W mercury lamp or 150 W xenon lamp ORTHOPLAN: Lamp housing 500 with 200 W mercury lamp, 150 or 450 W xenon lamp	ORTHOLUX II: Mirror housing 250/0 DIALUX: Lamp holder ORTHOPLAN: Mirror housing 60 S (incident light) Mirror housing 500					

Fluorescence Microscopes for transmitted-light darkground/ transmitted-light darkground fluorescence

Microscope	Light source	Base Mirror housing	Filter	Condenser	Objectives	Tube	Eyepieces
DIALUX Routine	Lamp housing 100 Z with 50 W mercury lamp Lamp housing 250 with 200 W mercury lamp	Base L	BB	Darkground condenser D 0.80 or D 1.20	Achromats, fluorite systems,	S	PERIPLAN 6.3x PERIPLAN GF 10x
ORTHOLUX II ORTHOLUX ORTHOPLAN Routine	Lamp housing 100 Z with 50 W mercury lamp Lamp housing 250 with 200 W mercury lamp ORTHOPLAN: Lamp housing 500 with 200 W mercury lamp	ORTHOLUX: Base A with mirror housing 30 S ORTHOLUX II: Mirror housing 250/0 ORTHOPLAN: Mirror housing 250 S Mirror housing 500	BB	Darkground condenser D 0.80 or D 1.20	Achromats, fluorite systems	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x ORTHOPLAN: PERIPLAN GW 6.3x GW/GG 8x, GW 10x
Research	Lamp housing 250 with 200 W mercury lamp or 150 W xenon lamp (FITC) ORTHOPLAN: Lamp housing 500 with 200 W mercury lamp, 150 or 450 W xenon lamp	ORTHOLUX: Base A with mirror housing 30 S ORTHOLUX II: Mirror housing 250/0 ORTHOPLAN: Mirror housing 250 S Mirror housing 500	BB SA	Darkground condenser D 0.80 or D 1.20	Achromats, fluorite systems, apochromats	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x ORTHOPLAN: PERIPLAN GW 6.3x GW/GG 8x, GW 10x

Fluorescence Microscopes for incident-light fluorescence/transmitted-light phase contrast

Microscope	Light source	Base Mirror housing	Filter	Condenser	Objective	Tube	Eyepieces
DIALUX ORTHOLUX ORTHOLUX II ORTHOPLAN Routine	Lamp housing 100 Z with 50 W mercury lamp Lamp housing 250 with 200 W mercury lamp ORTHOPLAN: Lamp housing 500 with 200 W mercury lamp	DIALUX: Lamp holder ORTHOLUX: Base A with mirror housing 30 S ORTHOLUX II: Mirror housing 250/0 ORTHOPLAN: Mirror housing 60 S (incident light) Mirror housing 500	BB	Fluorescence vertical illuminator and phase contrast condenser according to Heine or Zernike	Pv or PHACO phase contrast objectives	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x
DIALUX ORTHOLUX ORTHOLUX II ORTHOPLAN Routine	Lamp housing 100 Z with 100 W mercury lamp or 75 W xenon lamp Lamp housing 250 with 200 W mercury lamp 150 W xenon lamp (FITC) ORTHOPLAN: Lamp housing 500 with 200 W mercury lamp or 150 or 450 W xenon lamp	DIALUX: Lamp holder ORTHOLUX: Base A with mirror housing 30 S ORTHOPLAN: Mirror housing 250/0 ORTHOLUX II: Mirror housing 60 S (incident light) Mirror housing 500	BB SB SA	Fluorescence vertical illuminator and phase contrast condenser according to Heine or Zernike	Pv or PHACO phase contrast objectives	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x

Fluorescence Microscopes for incident-light fluorescence/transmitted polarized light

(Routine and Research)

Microscope	Light source	Base Mirror housing	Filter	Condenser	Objectives	Tube	Eyepieces
ORTHOLUX Routine	Lamp housing 100 Z with 50 W mercury lamp	Base A with mirror housing 30 S	BB and polarizer in holder and analyser in push-on mount	Fluorescence vertical illuminator and brightfield condenser series 600	Polarized-light objectives	FSA	PERIPLAN 6.3x PERIPLAN GF 10x
Research	Lamp housing 100 Z with 100 W mercury lamp Lamp housing 250 with 200 W mercury lamp	Base A with mirror housing 30 S	BB SB and polarizer in holder and analyser in push-on mount	Fluorescence vertical illuminator and brightfield condenser series 600 (if necessary also polarized-light condenser)	Polarized-light objectives	FSA	PERIPLAN 6.3x PERIPLAN GF 10x

Fluorescence Microscopes for simultaneous incident- and transmitted-light excitation

(Routine and Research)

Microscope	Light source	Base Mirror Housing	Filter	Condenser	Objectives	Tube	Eyepieces
ORTHOLUX ORTHOPLAN Routine	Transmitted and incident light: Lamp housing 100 Z with 50 W mercury lamp Lamp housing 250 with 200 W mercury lamp ORTHOPLAN: for transmitted light: Lamp housing 250 on the mirror housing 500 with 200 W mercury lamp for incident light: 200 W mercury lamp in the lamp housing 500	ORTHOLUX: Base A with 2 mirror housings 30 S For transmitted light: Mirror housing 60 ORTHOPLAN: in transmitted light: Mirror housing 250 S in incident light: Mirror housing 60 S	BB SA	Fluorescence vertical illuminator and darkground condenser D 0.80 or D 1.20	Achromats, fluorite systems	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x
Research	Incident light: Lamp housing 100 Z with 100 W mercury lamp or 75 W xenon lamp Transmitted light: Lamp housing 250 with 200 W mercury lamp or 150 W xenon lamp ORTHOPLAN: for transmitted light: Lamp housing 250 with 200 W mercury lamp or 150 W xenon lamp on mirror housing 500 for incident light: Lamp housing 500 with 200 W mercury lamp, 150 or 450 W xenon lamp	As under "Routine" Mirror housing 500	BB SB SA	Fluorescence vertical illuminator and darkground condenser D 0.80 or D 1.20	Achromats, fluorite systems, apochromats	FSA or FSA-GW	PERIPLAN 6.3x PERIPLAN GF 10x

D Objectives

All objectives which because of their large aperture concentrate much exciting light on the specimen or produce a high yield of fluorescence light are particularly suitable. The intensities increase as the square of the apertures.

Example: With objectives of a given primary magnification but different apertures of 0.50 and 1.00 the fluorescent light yield of the objective of larger aperture is about 4 times as much as that of the smaller aperture objective.

Oil- or water immersion objectives are preferable. Here no loss of light owing to refraction on glass/air interfaces and no reflections between coverglass and front lens of the objective occur.*

For the simultaneous methods suitable phase contrast objectives or polarized light objectives must be used. Generally all objectives can be used in fluorescence microscopy. It must, however, be borne in mind that objectives of low magnification do not produce a very bright fluorescent image. They should therefore be used only for occasional low-power observation.

* Water immersion objectives have the advantage that the water can be removed from the specimen quickly and completely and does not leave a greasy film behind.

The following objective combinations are particularly recommended:

Transmitted-light fluorescence

10/0.25
20/0.50
40/0.65
63/0.85
With adapter
PLEZY-FLU
Oel 100/1.30
with funnel stop 513362

Pl Apo 16/0.40
Apo 25/0.65
With adapter
PLEZY-FLU
Pl Apo 40/0.75
Apo 63/0.95
with adapter
PLEZY-FLU
Oel 100/1.30
with funnel stop 513362

Incident-light fluorescence

Apo 25/0.65
Fl 40/0.85
Apo 63/0.95
Fl Oel 95/1.32
or Iris Oel 100/1.30–1.10

Oel + W 22/0.65 with adapter PLEZY-FLU*)
Fl Oel 54/0.95 with adapter PLEZY-FLU*)
Pv Fl Oel 70/1.15 with adapter PLEZY-FLU*)
Iris Fl Oel 95/1.32–1.10 or for QM chromosome fluorescence according to Caspersson
Oel 100/1.30
with funnel stop 513362

*) the adapter is required only if objectives are used together with Oel 100 objective on the nosepiece.

In addition the following water immersion objectives will shortly be available:

W 25/0.60
W 50/1.00
W 100/1.20

They are particularly recommended for incident-light fluorescence.

E Eyepieces

The intensity of the fluorescent light depends on, among other factors, the magnification of the eyepieces. It decreases as the square of the eyepiece magnification increases.

Example: Eyepiece PERIPLAN 6.3x – intensity 100 %
Eyepiece PERIPLAN GF 12.5x – intensity 25 %.

Eyepieces of low magnification are therefore preferable for visual observation.

For fluorescence photomicrography (see Section III), however, the PERIPLAN GF 10x M or PERIPLAN GF 12.5x MF eyepieces with graticule for outlining the formats should be used for focusing.

III Fluorescence Photomicrography

A The specimen should meet the following conditions:
Clean preparation without dust particles and air bubbles; if possible no surplus dye in the interstices between fluorescent areas of the specimen (careful rinsing of the specimen during preparation, embedding in non-fluorescent medium);
Fluorescence as brilliant as possible:
Good differentiation of types of cell and detailed structures.

B The Microscope
The quality of the light source is important (before it is taken into use the lamp should be run for a day or longer; it will then burn more steadily with hardly any flicker).
When the intensity appreciably decreases or noticeable flickering occurs replace the lamp even before the end of its rated life.
Good centration of the light source and the condenser.
If possible use an objective of large aperture (immersion systems such as the Fl Oel 54/0.95). The image brightness increases as the square of the objective aperture.
For visual observation choose eyepieces of the lowest possible magnification (image brightness decreases as the square of the eyepiece magnification increases). For fluorescence photomicrography eyepieces of high magnification and with graticules outlining the format are required (PERIPLAN GF 10x M, GF 12.5x MF).
If the FSA tube is used (beam 100 % or 20 % directed to the eyepiece, 80 % to the photographic device) it is recommended to focus the specimen in the eyepiece always with full light intensity due to the sometimes low brightness.
For fluorescence photo micrography of weakly fluorescent specimens the monocular tube is particularly suitable: 100 % of the light is directed to the photo tube during photography, and 100 % to the eyepiece during observation.
Remove carefully dust from all glass surfaces in the beam path.

C The Camera
The following cameras are particularly well suited: The ORTHOMAT® automatic microscope camera with integrating and detail measurement for 35mm photography.
The large-format camera with the fully automatic exposure control for the 9 x 12cm (4 x 5in) format.
For brightly fluorescent specimens the micro-attachment for the LEICA®, the system attachment camera and the camera with POLAROID* CB 100 back are suitable; precise exposure measurement is carried out with the MICROSIX®-L exposure meter. If its measuring range is inadequate, an exposure series should be taken, if necessary.
For the accurate determination of the exposure time the measuring field of the exposure meter or of the camera should be completely occupied by a specific portion of the specimen.
If there is a tendency of underexposure because of the bleaching-out of small particles set the object ratio of the measuring field (e.g. ORTHOMAT) at 80–100 %.

® = Trade mark of Polaroid GmbH.

D The Exposure Material

For fluorescence photomicrography daylight colour films are to be preferred to black-and-white films because of their reproduction of the specific colour of fluorescence*. The results can be compared and reproduced because of the standardized development of colour reversal films.

Reproduction from colour originals is also recommended for black-and-white reproduction in print. Colour enlargements can also be obtained from colour transparencies.

Colour negative films are not recommended because of their generally low sensitivity and the subjectivity of positive production. Usually processing is carried out by processing stations where the technicians are unfamiliar with the fluorescence phenomenon. If these films are used nevertheless, a suitable transparency should, if possible, be included with the film when it is sent to the processing station to make colour comparisons possible.

Black-and-white films should be developed for good contrast.

* For the photography of blue fluorescent colours (e.g. catecholamine/neurotransmitter fluorescence), however, artificial-light colour films should be used because of their superior blue rendering.

The following exposure material was used in our Applied Microscopy Laboratory:

Black-and-white films

Manufacturer	Type of film	Speed (DIN)	Speed (ASA)	Remarks
Agfa-Gevaert	Scientia 50 B 65	18	50	
Kodak	Plus-X	22	125	
	Tri-X	27	400	very good contrast and fine grain
Ilford	Pan F	18	50	
	FP 4	22	125	
	HP 4	27	400	

Colour films

Agfa-Gevaert	Agfachrome professional 50 S	18	50	daylight film
	Agfachrome professional 50 L	20	80	artificial-light film; optimum blue rendering (e.g. catecholamine fluorescence)
	CT 18	18	50	daylight film
Kodak	Ektachrome High Speed	23	160	daylight film; special development when the speed was set at 27 DIN (400 ASA)
GAF	Ansco-chrome 500	28	500	daylight film; recommended for the photography of extremely small objects (e.g. spirochaetes), since the fluorescence intensity quickly decreases owing to bleaching
ORWO	UT 21	21	100	daylight film

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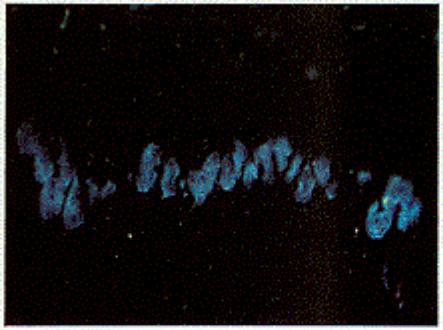
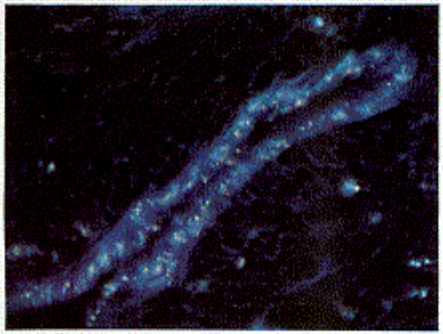
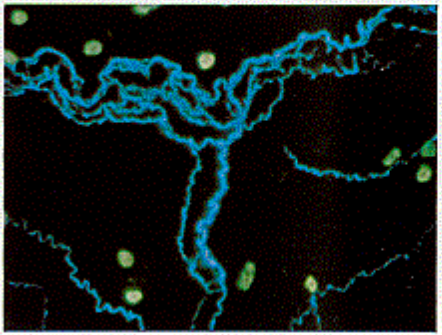


1
Muscle, snail
Catecholamine
Fl Oel 54/0.95
216x
Incident-light
fluorescence
BG 3 + S 405, K 460

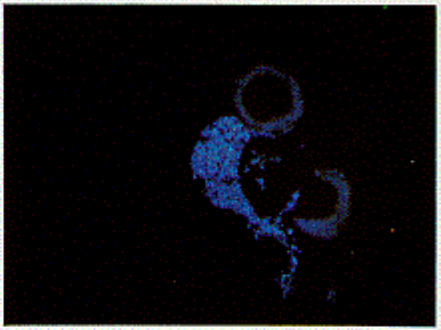


2
Muscle, snail
Catecholamine
+ 5-HT, otherwise
as Fig. 1

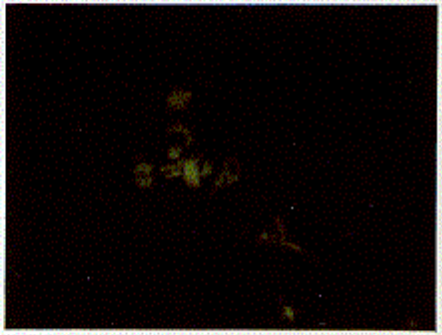


3
Mesentery, rat
Catecholamine
Pl 16/0.40
64x
Incident-light
fluorescence
BG 3 + S 405, K 460

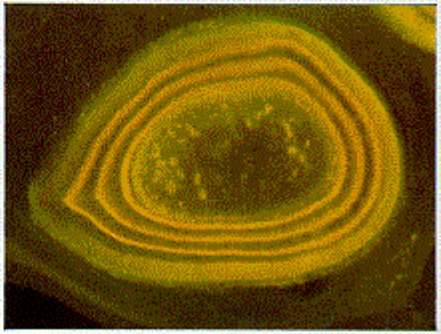
4
Eosinophilia
Aniline blue/
acradine orange
Oel 100/1.30
with funnel stop
400x
UG 1, K 430
Incident-light
fluorescence

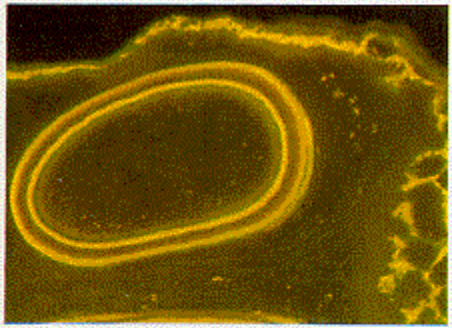


5
Streptococci
Group B (negative
demonstration)
Apo Oel 90/1.40
360x
Incident-light
fluorescence
BG 12 + S 470/K 510

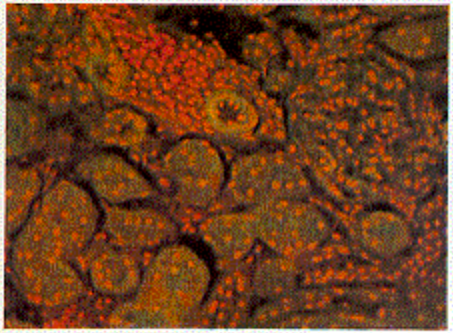


6
Tooth, Pomeranian
Tetracycline
Pl Fl 4/0.14
16x
Transmitted-light
fluorescence
BG 12, K 530

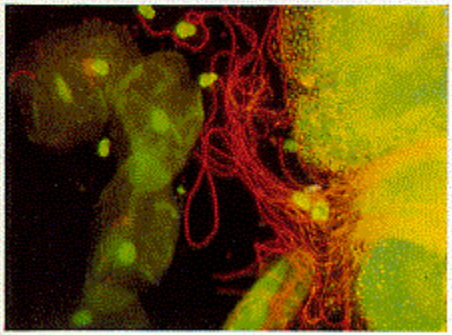




7
Tooth, Pomeranian
Tetracycline,
otherwise as Fig. 6

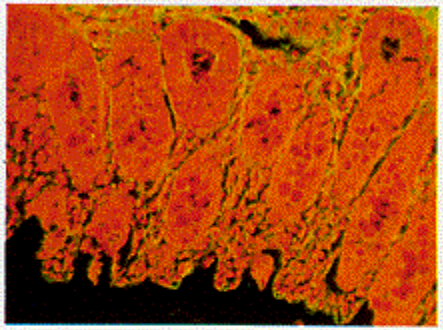


8
Kidney, rat
Acridine orange
25/0.50
100x
Transmitted-light
fluorescence
BG 12, K 530

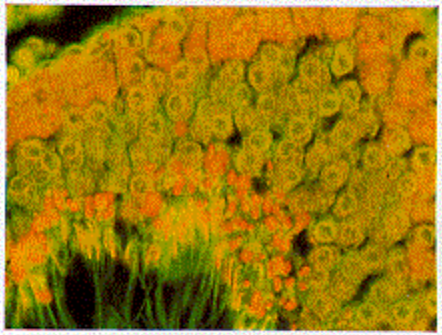


9
Bacteria on
Epithelial cells of
the buccal mucosa
Acridine orange
40/0.65
160x
Transmitted-light
fluorescence
BG 12, K 530

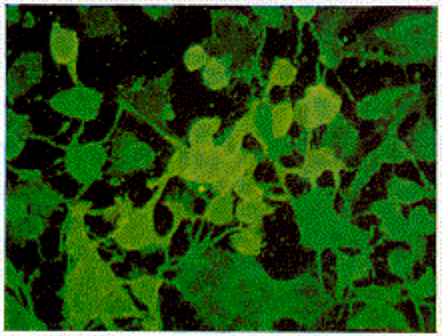
10
Intestine,
guinea pig
Acridine orange
Oel + W 22/0.65
88x
Transmitted-light
fluorescence
BG 12, K 530

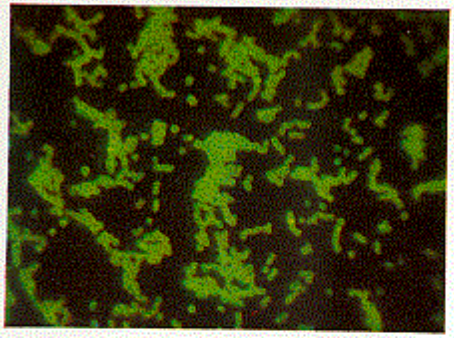


11
Testicle, rat
Acridine orange
Fl Oel 54/0.95
216x
Incident-light
fluorescence
BG 12, K 530

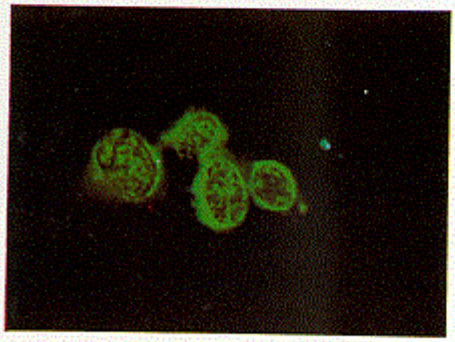


12
FITC demonstration
of measles
Oel + W 22/0.65
88x
Incident-light
fluorescence
2x KP 490, S 525

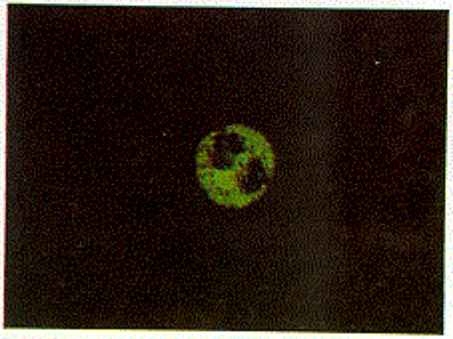




13
Streptococci
Group B (positive
demonstration)
Apo Oel 90/1.40
360x
Incident-light
fluorescence
BG 12+S 470/K 510

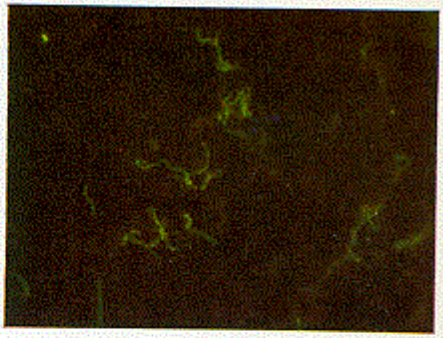


14
FITC demonstration
of German measles
Fl Oel 54/0.95
216x
Transmitted-light
fluorescence
KP 490, K 510

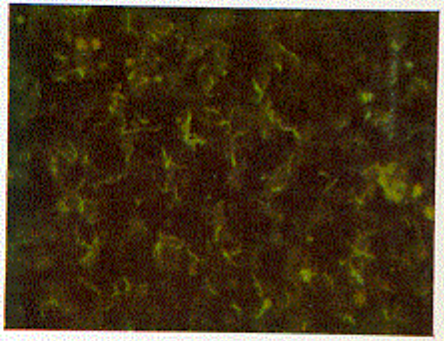


15
Gonococci (positive
demonstration)
in pus cell
Oel 100/1.30
400x
Incident-light
fluorescence
BG 12+S 470/K 510

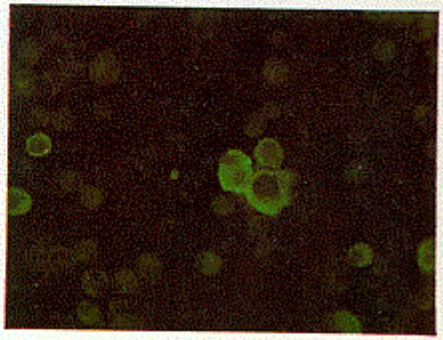
16
FTA-test
FITC
Apo Oel 90/1.40
360x
Incident-light
fluorescence
2x KP 490, K 510

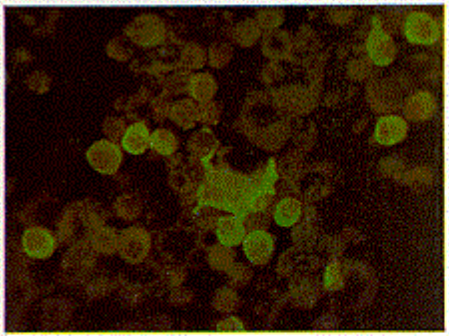


17
FTA-test
FITC
Fl Oel 54/0.95
216x
Incident-light
fluorescence
2x KP 490, K 510

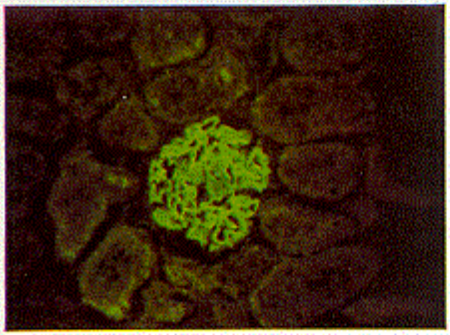


18
Bone marrow cells
FITC
Fl Oel 54/0.95
216x
Incident-light
fluorescence
2x KP 490, S 525

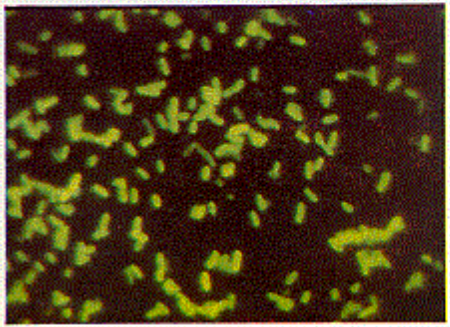




19
Bone marrow cells
FITC
Fl Oel 54/0.95
216x
Incident-light
fluorescence
2x KP 490, S 525

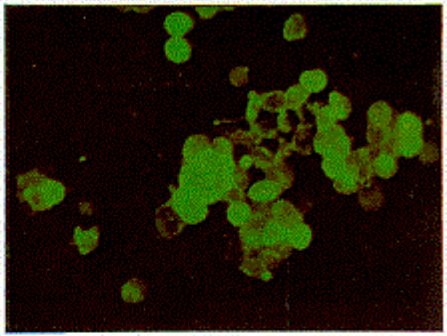


20
Kidney glomerulus
FITC
25/0.50
100x
Transmitted-light
fluorescence
BG 12, K 510

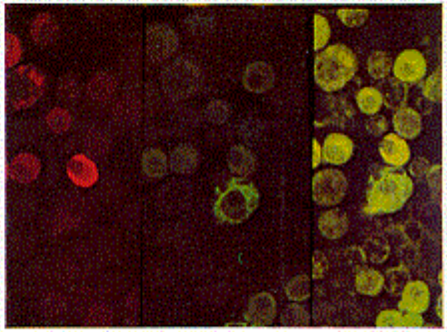


21
Bacteria coli
FITC
Fl Oel 95/1.32
380x
Incident-light
fluorescence
BG 12, K 510

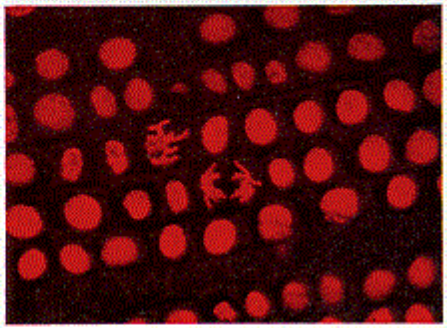
22
FITC demonstration
of measles
Oel+W 22/0.65
88x
Transmitted-light
fluorescence
BG 12, S 525



23
Bone marrow cells
(double labelling
with FITC and
TRITC)
Fl Oel 54/0.95
216x
Right:
2 KP 490/K 510
Centre:
2 KP 490/S 525
Left:
S 546+BG 36/K 610



24
Onion root
Feulgen
Fl Oel 54/0.95
216x
Transmitted-light
fluorescence
S 546+BG 36, K 610



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